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Enzymes of the Central Nervous System

Six-Year Medical Course

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ENZYMES OF THE CENTRAL NERVOUS SYSTEM

BY

HUBERT MORTON ENGLISH

THESIS

FOR THE

DEGREE OF BACHELOR OF ARTS

IN

Six Year Medical Course
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IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

DEGREE OF BACHELOR OF ARTS

IN THE SIX-YEAR MEDICAL COURSE

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ENZYMES OF THE CENTRAL NERVOUS SYSTEM

I PURPOSE.

The investigations, the results of which are recorded in the following pages, were undertaken with the aim of determining as far as possible in the time allotted, which enzymes are present in the central nervous system; their distribution in the different divisions of the brain; and their chemical properties. A number of different methods have been used in preparing the tissue extracts, but even if a particular enzyme could not be detected by any of these methods, its presence is not entirely disproved. In studying the chemical properties, no attempt has been made to prepare an enzyme in a pure state, but the experiments have been performed with a view of finding out the best conditions under which the enzymes act. The conditions which have been varied are the acidity or alkalinity of the solution, the relative proportions of enzyme and substrate, the amount of dilution, compounds which accelerate the reaction between enzyme and substrate, and the time during which the enzyme was allowed to act.

The importance of an investigation of this kind rests principally upon the possibility of the application of its results in the therapy of abnormal nervous conditions. In a number of diseases of the nervous system, it is a known fact that certain enzymes are present in abnormally large quantities. Whether the overabundance of a particular enzyme produces the diseased condition, or is merely concomitant with it, is a question for pathologists. But, at any rate, it seems reasonable to assume that in some diseased conditions of the nervous system, certain enzymes may be present in abnormally small quantities, on even entirely

absent, since large quantities of enzymes are present under certain abnormal conditions; and in either case, a knowledge of the enzymes normally present would doubtless be of considerable aid in the medical treatment of the condition.

Investigational work, however, need not necessarily be of immediate practical application in order to be important. It often happens that facts are used in a practical way years, or even centuries, after they have been discovered, and hence, since no one can tell what facts are to be of practical value in the future, any effort which aids in getting facts out of the realm of mystery, is of importance.

II HISTORICAL.

A number of enzymes have already been detected in nervous tissue. In fact, most enzymes known to exist in the animal body have been shown to be present in the nervous system, and the theory that the enzyme content of one tissue is quantitatively and not qualitatively different from the enzyme content of other tissues, has received confirmation from the investigations on nervous tissue. For instance, rennin,[1] an enzyme usually thought of only in connection with the curding of milk in the stomach, has been shown to be present in many parts of the body, among them the brain.

However, work has not always given clear, definite results and tests for the same enzyme, in the hands of different investigators, have given conflicting data. That brain does contain a protein splitting enzyme, as stated by Kutscher and Lohman[2] has been confirmed since by many men. Vernon[5] detected erepsin in the brain as well as in many other tissues. Buetow,[18] in working with the hypophysis, found pepsin and trypsin, and other investigators also have reported a proteolytic enzyme. Negative results have been obtained, however, by Wroblewski[10] and others. An enzyme capable of splitting glycyltryptophane has been found in normal cerebro-spinal fluid by Wohlgemuth and Szecsi,[15] and they further state that this enzyme is abnormal in the brain.

A lipase, which splits lecithin and kephalin as well as fats, has been demonstrated by many authors and has been found absent only by the earlier investigators. Kutscher and Lohman[2], in studying the autodigestion of the brain, were unable to detect cholin in the solution, and concluded from this that no lecithin-splitting enzyme was present. Pagenstecher,[7] however, working a

little later on the presence of lipase in various tissues, found it in the brain, and his work has been confirmed since by many investigators, among them Wroblewski,[10] Coriat,[4] and Buetow.[18] The lipase has also been shown to be present in normal cerebro-spinal fluid and in both brain and cerebro-spinal fluid under abnormal conditions. No distinction has been observed between the fat-splitting and lipoid-splitting power of the tissue extracts, although one would expect to find a different enzyme for each class of fatty compounds, just as there are different diastases and different proteases. Apparently the same enzyme splits all fats and fat-like substances, but acts with different rates on different substrates.

The amylolytic power of brain tissue is more open to question. An enzyme capable of breaking down starch into dextrin was reported by Wroblewski,[10] who believed that it was present for the purpose of acting on glycogen. The same author using water extracts of the brain, also obtained action on the glucosides, arbutin and salol. Whether or not a single enzyme splits starch, arbutin and salol is uncertain, but it seems probable, because of the complex nature of starch, that a separate enzyme attacks it, while the arbutin and salol may be acted upon by one enzyme. A diastase was also detected in the hypophysis by Buetow[18] who further states that this diastase is not lactase or invertase. Diastase has been reported by Wohlgemuth and Szecsi[15] in normal cerebro-spinal fluid, but the same authors were unable to detect it in the brain. Because of the relation of brain and cerebro-spinal fluid, one would expect to find the same enzymes present in both, and the fact that these authors failed to find diastase in the brain, may be explained on the ground that it is present there in much smaller quantities than

in the fluid.

It is interesting to note here that the diastase content is considerably increased during certain pathological conditions. Thus, Sieburg[14] found diastase and a glucocide-splitting enzyme in the hydrocephalus fluid of a thirteen months-old boy and Kafka[16] reports diastase in large amounts in paralytics, alcoholics and in cases of dementia praecox, arteriosclerosis, senil processes and acute meningitis, the more advanced cases showing the greater content. The latter author found little or no diastase in normal brain. From these and other investigations, it would appear that the diastase content of brain and cerebro-spinal fluid is normally very small while it is increased by many abnormal conditions.

The presence of catalase in brain tissue does not seem to be denied by any authors who have investigated the subject. Practically all parts of the body have been shown to possess the power of decomposing hydrogen peroxide. This enzyme has been reported absent, however, from normal cerebro-spinal fluid.

The presence of peroxidase in brain tissue has been reported by Levene and Stookey[3] and confirmed by numerous investigators since. Vernon[8] found an indophenol oxidase in many tissues, and states that the amount present runs parallel with their degree of oxygen saturation. Hence the brain was found to be relatively rich in oxidase in comparison with the lung, liver, and other tissues.

An enzyme which decomposes urea has been observed in the hypophysis by Buetow,[18] but negative tests for a deaminase were obtained by the same author.

III DESCRIPTION OF METHODS USED.

The material used in these experiments has been obtained from various sources. Beef brains were obtained from Dallenbach Bros. of Champaign, as soon as possible after the animals were slaughtered. These were transported in ice to the laboratory and used immediately, so that not more than four or five hours elapsed from the time of the death of the animals to the beginning of extracting, freezing,or whatever process was first used.

Dog brains were obtained immediately after death from dogs experimented upon in the Physiology department and were treated as were the beef brains.

One human brain was obtained from Chicago, sent packed in ice.

The greater part of the experiments, however, have been carried out on sheep brains obtained from Armour and Co. of Chicago. These were always sent well surrounded with ice and were received in excellent condition. They had at least two distinct advantages over the brains obtained from the local supply. In the first place, they were not so badly mangled, so that more accurate separations of the different divisions of the brain could be made, and in the second place they did not contain so much blood. The brains obtained from Chicago had the possible disadvantage of being longer out of the animals before being used, but the activity obtained from them was apparantly as large as was obtained from the other brains.

In all cases the brains were deprived of their membranes. The brains containing blood clots were washed to remove as much of the blood as possible. Where the comparison of the different divi-

sions was to be made, the brain was next divided into cerebum, cerebellum, medulla, mid-brain and corpus callosum. These different divisions were minced separately in a meat chopper and then ground to a fine state in a mortar with either toluol or oil of mustard as a preservative, usually the latter.

Direct extractions of the cerebra were made with water, .2% HCl, .9% NaCl, .5% Na_2CO_3, a saturated solution of choroform in water, and glycerin. In each case, one cubic centimeter of the liquid was used for each gram of brain. The other divisions of the brain were extracted with water, the same proportions being used.

Other tissues were dried before extraction by two different methods. In one, the freezing method, the ground tissue was spread in thin layers on watch glasses and placed in desiccators. Surrounding the watch glasses, was a layer of calcium chloride which was held between a cylindrical screen and the wall of the desiccator Calcium chloride was also placed in the bottom of the desiccator and in a dish on top of the tissue. The desiccator was then put into a jar and ice packed around it. After sufficient time had passed for the tissue to be frozen, evacuation of the desiccator was begun. Tissue could be dried by this method in from $\frac{3}{4}$ to 2 days.

In the second drying method, the ground tissue was spread in thin layers on glass plates and placed in a specially constructed box, through which a current of air was drawn by means of an electric fan. An electric heating apparatus, at the opposite end of the box from the fan, could be used to warm the air passing over the tissue; but since it was found that, by repeated turning, the tissue could be dried in from ten to twenty hours without heating the air, this apparatus was not used.

When tissue which had been dried by either of these methods was extracted, one c.c. of extractive was used for each one-fifth gram of tissue, since nerve tissue contains approximately eighty per cent water.

Some of the tissue dried by the air current was extracted with benzene to remove the lipoids, before extraction of the enzyme. The amount of dried tissue was weighed and placed in a bottle with benzene, and shaken on a mechanical shaker for one to two hours. The benzene was then filtered off and a second portion added and shaken as before. Practically all of the lipoids were removed and a light, powdery substance left. When this was to be extracted for the enzymes, its weight before extraction with benzene was multiplied by five for the weight of the original tissue, and one c.c. of extractive added for each gram of original tissue.

In making the extractions, the tissue was placed in cheese cloth sacs and suspended in wide-mouthed bottles containing the extractives. More preservative was added and the bottles stoppered. These were left at room temperature for from three to seven days. The sacs were then lifted out of the extractive, allowed to drain for a few hours, and discarded. The extracts were set away in a refrigerator and used as needed.

The test solutions were ordinarily prepared as follows: .4 c.c. of substrate was added to 10 c.c. of extract and 100 c.c. of water. The solution was mixed thoroughly and 50 c.c., by accurate measurement with a pipette, were placed in each of two 150 c.c. ehrlenmeyer flasks. One of these was boiled to destroy the enzyme, and both were then placed for 48 hours in a thermostat kept at 37.5°. When a large number of tests was to be run on the

same substrate, a solution was made containing the right proportions of substrate and water, and 100 cc of this solution used for 10 c.c. of extract. In the case of insoluable substrates, like olive oil and ethyl butyrate, emulsions of these were produced by making the liquids slightly alkaline and thoroughly shaking. When activators were used, they were always added to the enzyme, since Rosenheim and Shaw-Mackenzie[20] have shown that they are not effective unless added before the enzyme has a chance to combine with the substrate.

After forty eight hours in the thermostat, the amount of activity in the tubes was determined. For the action on fats, fatty compounds and salol direct titration was made with tenth normal sodium hydroxide, using phenolphthalein as an indicator. For the action on proteins, the formaldehyde method was used. With anylases the amount of activity was estimated by the copper hydroxide test.

IV a. RESULTS.

It was desirous at the outset to find the best method of extracting the enzymes or, at least, a good method. It is obvious that the method which might prove best for one enzyme, might be a very poor method for extracting others. Active lipolytic extracts were obtained with all the solvents used. Consequently, in order to find the best method, a series of extractions was made on the cerebum portion under as nearly as possible identical conditions. Two such series, after extracting for seven days, gave the following results when acting on a mixture of equal amounts of monoacetin and monobutyrin.

Tissue	Extractive	$\frac{N}{10}$ NaOH required for neutralization.	
Fresh Cerebrum	Water	1.34 c.c.	.85 c.c.
" "	.2% HCl	.13 c.c.	.24 c.c.
" "	.9% NaCl	1.21 c.c.	.82 c.c.
" "	.5% Na_2CO_3	1.30 c.c.	.70 c.c.
" "	$CHCl_3$ water	1.34 c.c.	1.13 c.c.
" "	Glycerin	3.75 c.c.	.78 c.c.
Frozen " no preservative	H_2O	1.94 c.c.	
" "	Glycerin, no preservative	.11 c.c.	
" " with preservative	H_2O	2.31 c.c.	
Air dried "	H_2O	1.37 c.c.	
" " " (Extract with benzene)	H_2O	2.71 c.c.	

Another series of extractions was run on the cerebrum with a view of finding out the rate of extracting of the various liquids. In this series ethyl butyrate was used as a substrate.

		$\frac{N}{10}$ NaOH required for neutralization after extracting for			
Tissue	Extractive	5 days	7 days	9 days	12 days
Cerebrum	H_2O	.30 cc	1.25	1.18	1.83
"	.2% HCl	.19 "	.85	.50	.57
"	.9% NaCl	.38 "	.60	1.10	1.19
"	.5% Na_2CO_3	.45 "	.64	1.30	1.18
"	$CHCl_3$ solution	.66 "	.56	.39	1.24
"	Glycerin	.15 "	.14	.37	.50

The results of these experiments indicate that water sodium chloride, sodium carbonate and chloroform water are about equal as extractives. In one case the values are 1.34, 1.21, 1.30 and 1.34 respectively and in the other .85, .82, .70 and 1.13 respectively, when acting on monoacetin and monobutyrin. But when acting on ethyl butyrate,the activity of the water extract is lower at the end of five days, but from then on, it rises more rapidly and is the largest at the end of twelve days. The acid extractive seems to be less well suited for extracting the lipase, since the values for its activity are considerably lower. In the second table, the chloroform water seems to extract the enzyme more rapidly at first, but the decline in activity from the fifth to the ninth day and the rapid rise at the end of the twelf day is unaccounted for.

In the two tables the glycerine presents conflicting results. In the first experiment, it gave the greatest activity of any extractive and in the second, its activity was high, in both cases acting on monoacetin and monobutyrin. In the second

table, however, its activity is lower than that of any other extractive.. This difference is doubtless due to the fact that in the second table, ethyl butyrate was used as a substrate.

The drying method, in general, seems to give slightly better results than the direct extraction method. The air dried, extracted immediately with water, gave about the same activity as the fresh tissue extracted with water, the figures being 1.34 and 1.37. The air dried tissue which had been extracted with benzene gave the highest activity of any of the dried portions. Just why this is true, it is hard to say, unless the lipoids exert an inhibiting influence upon the lipase.

With the frozen tissue extracted with water, greater activity was obtained than with fresh tissue extracts. The tissue frozen without a preservative gave a little less activity than that in which a preservative was used. (A preservative was used in both cases when the extractions were made). It is doubtful if these fiqures are of any great significance, other than the fact that the preservative, which was oil of mustard in these series of experiments, exerted no inhibiting influence upon the enzyme. The frozen tissue which was extracted with glycerin gave very little activity. Doubtless, this is to be explained on the ground that the glycerin mixed very slowly with the dry tissue. In the case where the fresh tissue was extracted directly with glycerin, the glycerin mixed fairly readily, since the tissue contains about 80% water which is readily miscible with glycerin. Had the dried tissue been extracted with equal parts of water and glycerin, larger activity doubtless would have resulted.

The different divisions of the brain when kept under

conditions as nearly as possible identical and extracted with water gave the following activity

	c.c. of $\frac{N}{10}$ NaOH	
	I	II
Cerebrum	1.34 c.c.	1.25
Cerebellum	2.10	.87
Mid-brain	1.25	1.23
Medulla	.45	.36
Corpus callosum	.61	.56

These results indicate clearly that the grey matter is more active from a lipolytic standpoint than the white. The corpus callosum, which is made up entirely of white matter, would naturally be expected to give the lowest activity, but although it was obtained as free as possible from grey matter, it nevertheless showed greater activity then the medulla. The activities of the mid-brain and cerebrum in each case were about equal, while the activityof one cerebellum was higher and of the other lower, than those of the cerebrum and mid-brain. Since the mid-brain contains large nuclei of grey matter, it seems probable that the greater part of its activity depends upon these. The medulla, which is very similar to the mid-brain structurally but lacks the large nuclei, showed considerably less activity.

The lipase found in different portions of the brain possesses the power of acting on many substrates. Some results obtained with different extracts are given in the following table.

c.c. of $\frac{N}{10}$ NaOH required for neutralization

	Glycerin extract of cerebrum	Water extract of cerebrum	Water extract of medulla	Na_2CO_3 extract of cerebrum
Triacetin	6.25		.56	.36
Monoacetin	8.20	1.37 2.80		.29
Tributyrin	4.25			.24
Monobutyrin	2.46 2.90	.84 2.40	.25	.08
Ethyl butyrate	4.40 2.35	2.23	.15	.13
Olive oil	4.17	.15 .26	.29	.56
Lard (hog)				.19
Tallow (beef)				.16
Butter	4.47			.17
Lecithin	3.35	.25 .19		.00
Kephalin	3.08	.20 .27		.23

The greatest activity was obtained from a glycerin extract of the cerebrum, although there is no basis for comparison, since the conditions under which the different experiments were carried on were varied. The water extract also gave good activity, while the Na_2CO_3 extract was only slightly active. The water extract of the medulla was weak in activity.

The best substrates as shown by these experiments, are triacetin and monoacetin. Tributyrin, monobutyrin and ethyl bytyrate, were acted upon quite readily. Lard, tallow and butter evidently owe their lesser activity to the fact that they do not go into solution, and at best form only finely divided particles when ground with water in a mortar or thoroughly shaken with water.

Since the activity at any one time depends upon a large number of conditions, it was decided to run a set of experiments in which each condition would be varied with the remaining condi-

tions. The different conditions varied were the amount of extract, the amount of substrate, the substance used as an activator, the amount of dilution, and the time during which the enzyme was allowed to act. Three different variations were chosen under each condition. The substrate used was ethyl butyrate.

		Extract in c.c.			Substrate in c.c.			Activators			Dilution in c.c.			Time in days.		
		2	5	15	0.1	0.2	1.	NaH_2PO_4 Na_2HPO_4	Sodium Glyco-cholate	Saponin	0	25	100	1	3	6
Extract	2				.19	.28	.41	.22	.12	.20	.28	.09	.10	.07	.31	.60
in c.c.	5				.26	.37	.53	.38	.31	.34	.49	.36	.44	.18	1.40	1.65
	15				.40	.64	1.25	.55	.20	.60	1.60	.44	.68	.28	1.60	2.15
Sub-	0.1							.20	.0	.15	.40	.30	.27	.10	.47	.68
strate	0.2							.30	.41	.34	.53	.44	.49	.19	.52	1.03
in c.c.	1.							.92	.53	.68	.77	1.30	.53	.61	.88	1.43
Activators	Na_2HPO_4 NaH_2PO_4										1.80	.57	.60	.25	.58	1.25
	Sodium Glycocholate										.22	.12	.34	.14	.48	.65
	Saponin										.35	.34	.50	.22	.63	.97
Dilu-	0													.30	.70	.68
tion.	25													.18	.45	1.05
in c.c.	100													.23	.84	1.29
Time	1															
in	3															
days	6															

In preparing the solutions in these experiments, a considerable amount of each proportion of ethyl butyrate and water desired, was made slightly alkaline and thoroughly emulsified. When used, the emulsion was thoroughly mixed with the extract and two equal amounts of the mixture taken, one for a check and the other for enzyme activity. In the cases where an activator was used, it was always mixed with the extract before either was added to the substrate. The figures in the results represent the c.c. of $\frac{N}{10}$ NaOH required to neutralize the difference between the check and normal flasks.

Where one set of conditions was varied with another, the remaining conditions were kept under circumstances found favorable in previous experiments. Thus, when the amount of extract was varied with the amount of substrate, no activator was used, the dilution was 50 c.c. and the time two days. When the amount of dilution was varied with the time, 5 c.c. of extract were used, .2 cc of substrate, and no activator.

The results indicate clearly that the amount of activity of the brain lipase on ethyl butyrate varies directly with the amount of enzyme, and the amount of substrate used. In every case with the exception of one the activity was increased where either one of these factors was enlarged. The one exception is the case of the variation of the amount of extract with sodium glycocholate as an activator. Here the greatest activity, contrary to expectations, was obtained in the flask containing 5 c.c. of extract, and not in the one containing 15 c.c.

With the activators, the results were less definite. The amount of phosphates used, was 2½ c.c. of a one per cent solution of

equal amounts of the monobasic and dibasic phosphates of sodium. $2\frac{1}{2}$ c.c. of a .02 molar solution of sodium glycocholate, and $2\frac{1}{2}$ c.c. of a .25 per cent solution of saponin, were used. The greatest activity was obtained with the phosphates and the least with the sodium glycocholate, although the activity was not increased markedly in any case. As is to be noticed from the table, it is doubtful if the activity was increased at all with sodium glycocholate. If any conclusion can be drawn, it would seem to be that sodium glycocholate does not markedly increase the activity of brain lipase on ethyl butyrate under any of the conditions under which it was tested.

With saponin, slightly higher activity was obtained, but this was not above the normal activity. The three values obtained for saponin, when the amount of extract was varied, were .20, .34 and 1.60. The three corresponding normal values were .28, .37 and .64. When the amount of substrate was varied, the three values were .15, .34 and .68, while the normal values were .26, .37 and .53. It would seem from these latter results that saponin has more influence in larger amounts of substrate, although the differences is not sufficient to guarantee this conclusion. The values obtained for the activity of saponin under different dilutions do not differ to any appreciable extent from the normal values. With the different lengths of time, the values are also close to normal. The value 1.40 obtained under normal conditions with respect to extract, substrate and dilution, but with the enzyme acting for three days, is abnormally high. The reason for this is not apparent.

The values obtained with a mixture of mono- and dibasic

phosphates acting on different amounts of enzyme are approximately equal to the normal values, and the amounts of activity obtained with the two smaller concentrations of substrate are not far from the normal values, but the value obtained with phosphates acting on a large amount of substrate is considerably higher than the corresponding normal value, the figures being .92 and .53 respectively. This result would seem to indicate that phosphates, like saponin, increase the activity in large concentrations of the substrate. This conclusion is further supported by the action in small amounts of dilution where phosphates are present. The values obtained under normal conditions for no dilution are .49 and .53, while with phosphates the value is 1.80. In the dilutions of 25 and 100 c.c. the values are only slightly above the normal values. The results obtained for different lengths of time through which the enzyme was allowed to act, are not markedly different from the values obtained when no phosphates were present.

In varying the different amounts of dilution a peculiar result was obtained. Activity was as a rule greater in both the smallest and largest dilutions than it was in the medium dilution. The only two marked exceptions to this were obtained with a large amount of substrate and with a long length of time. It can be conceived that one c.c. of substrate in 25 c.c. of dilution forms the most favorable combination for large activity by the amount of enzyme contained in 5 c.c. of extract. If this be true, then it follows that 1 c.c. of substrate with no dilution forms too concentrated a medium for the best action of the enzyme and in fact the result obtained under those conditions is considerably smaller. The other exception occurred in the test with small dilution, which

was allowed to act for six days, this result being low. This fact is doubtless to be explained on the grounds that in the small dilution the end point of activity, or point of equilibrum, was reached sooner than in higher dilutions. The values obtained at the end of three and six days were practically equal in the case of the small dilution, while in the higher dilutions, the amount of activity steadily increased.

In the flasks in which the time of action of the enzyme was varied with the other factors, the amount of activity gradually increased with the time, with one exception; in the small dilution, as mentioned above, the activity seems to have reached its heighth at the end of the third day. Otherwise, there was no evidence of the end-point-of-activity having been reached, even after six days.

In regard to the influence of activators on the action of the enzyme, it should be stated that larger increases in activity were obtained when monoacetin was used as a substrate with a water extract of the cerebrum. Values obtained under comparable conditions are as follows:

			c.c. $\frac{N}{10}$ - NaOH.
5 cc extract	.2cc monoacetin		2.87
" " "	" "	2½cc .02 M Sodium glycochlate	3.05
" " "	" "	2½cc .25% saponin sol.	3.11
" " "	" "	2½cc 1.% phosphate sol.	3.63

From these results it will be seen that sodium glycocholate and saponin slightly increased the activity of the lipase, while phosphates increase it markedly. Previous investigators[20] have shown shown that saponin and sodium glycocholate considerably increase the activity of the pancreatic lipase and Wroblewski[10] has

studied the accelerating action of phosphates on the brain lipase.

With regard to the nature of the medium in which the lipase acts best, experiments show that a medium of slight acidity is best suited to its activity. In testing out this point, alkaline extracts were used in two different experiments. In one flask, the solution was left alkaline, while in the other a slight acidity was produced. Similarly, acid extracts were tried out in both acid and alkaline media. With a mixture of monoacetin and monobutyrin, the following results were obtained:

				c.c. $\frac{N}{10}$ NaOH.	
.5% Na_2CO_3	extract	slightly	acid	1.15	.70
"	"	"	alkaline	.79	.37
.2% HCl	"	"	acid	.33	.24
"	"	"	alkaline	.17	.18

The alkaline extract as shown before is more active than the acid, but larger activity is produced by the alkaline extract if the medium is made slightly acid at the start. The acid extract, likewise, is better suited to an acid medium.

The presence of a proteolytic enzyme in nerve tissue has been proven by the action of tissue extracts on various proteins. Time did not permit of an extensive study of this enzyme, or enzymes; but it has been found to attack several different proteins. A glycerin-water extract of the cerebrum, prepared by the freezing method, gave the following results, when the resulting solution was tested for amino acids by the formaldehyde method.[21]

	c.c. $\frac{N}{10}$ $Ba(OH)_2$
Egg albumen	.2
Gelatin	1.92
Casein	.98
Witte's peptone	.95
Nuclein (water extract)	.69

The splitting of Witte's peptone is in accord with the work of Vernon[5] on the presence of erepsin in various tissues, but it would seem that erepsin alone would hardly be sufficient cause for the action on the more complex proteins, and it is probable that a pepsin or trypsin-like enzyme, or both, are present throughout the brain as well as in the hypophysis where both were found by Buetow.[18]

The proteolytic action of brain extracts on Witte's peptone, in further accordance with Vernon's work, takes place most rapidly in alkaline medium.

	c.c. $\frac{N}{10}$ $Ba(OH)_2$
Witte's peptone, slightly acid medium	.50
" " " alkaline medium	1.92

Tests of the various kinds of extracts on Witte's peptone gave the following amounts of activity, 5 c.c. of extract and 5 c.c. of a saturated solution of peptone being used in each case.

	$\frac{N}{10}$ $Ba(OH)_2$
Water	.15
.2% HCl	.12
.9% NaCl	.16
.5% Na_2CO_3	.25
$CHCl_3 \cdot H_2O$	.21
$C_3H_5(OH)_3$	.45

Here again the acid extract is shown to form a poor medium for the action of the protease. The sodium carbonate extract is better, but the highest activity was obtained with the glycerin extract and the results of a previous table show further that that extract was effective on other proteins as well as Witte's peptone.

It might be mentioned in connection with the work on proteolytic enzymes, that in searching for rennin, negative readings were obtained from the results of the metacasein' test.

The amylase content of brain tissue seems to be very slight. No action at all could be detected in tests on starch, glycogen, amygdalin, sucrose, maltose, lactose, and dextrose. Dextrin, however, was slightly hydrolyzed. A solution of dextrin in which the enzyme had been allowed to act, gave a slight copper hydroxide test for the presence of a reducing sugar, while the check solution gave no test. The glucosides, arbutin and salol, as shown by Wroblewski,[10] are hydrolyzed by water extracts of the brain. A solution of arbutin, after being acted upon by the enzyme, gave a strong copper hydroxide test, while in the boiled check no copper was reduced.

With salol, the following results were obtained, by direct titration of the solutions.

	c.c. $\frac{N}{10}$ NaOH
Unboiled flask containing enzyme and salol	1.52
Boiled " " " " "	.76
Difference = enzyme activity	.76

Direct titration is possible since salol, upon hydrolysis, yields phenol and salicylic acid.

$$C_6H_4OH - CO - OC_6H_5 + H_2O = C_6H_4OH - COOH + C_6H_5OH$$

Salol salicylic acid phenol.

Since the cerebroside, cerebrin,contains a galactose grouping, it was thought possible that this might be split off by the amylase, but in several tests made on a mixture of cerebrosides, no reduction of copper was obtained.

Catalase can easily be proved present in brain tissue. A water extract of the cerebrum was divided into two portions, one of which was boiled. Equal amounts of these two solutions were then mixed with equal amounts of hydrogen peroxide, were placed in fermentation tubes, and set in the thermostat. At the end of two hours, the tube containing unboiled solution had in it from four to eight cubic centimeters of gas which proved to be oxygen, while the boiled check had produced no gas. The results show that the decomposition of hydrogen peroxide, which takes place at a slow rate under normal conditions, is greatly accelerated by the presence of the brain extract, and since such acceleration is not produced by boiled extract, it must be due to some thermolabile compound contained in the extract.

Tests were made for other enzymes and negative results obtained. Peroxidase was tested for by the guaiac test, in which guaiac, in the presence of hydrogen peroxide, is turned blue if the enzyme is present. No blue coloration was obtained. Tests for oxidase also resulted in the negative. A solution of alpha naphthol was not changed to a blue color in the presence of a tissue extract, as is the case when certain oxidases are present; formaldehyde was not oxidized to formic acid; and xanthin and hypoxanthin were not converted into uric acid. It is probable, since both of these enzymes have been found by several investigators, that conditions were not exactly favorable for their detection or

the specific tests were not used. It has been shown that glucose[22] and other powerful reducing substances, haemoglobin,[23] and protein[30] interfere with different oxidase tests.

Reductase could not be demonstrated by the methylene blue test which depends upon the decoloration of a dilute solution of methylene blue by formaldehyde. Here again the conditions may have been unfavorable for the action of the enzyme.

Guanase, which transforms guanine into xanthine, and urease, which produces carbon dioxide and water from urea, could not be detected in the brain tissue extracts. It is probable that both of these, if present at all, are present in such small quantities, as to be undetectable by the methods used.

Human, beef, sheep and dog brains have been tested for lipolytic activity and all found to be active. No experiments were run with extracts from each source under strictly comparable conditions, so that no estimate could be made of the relative quantities of lipase contained in the brain from the different sources. Figures which were obtained from extracts of the different brains acting on triacetin, are given below.

	c.c. $\frac{N}{10}$ NaOH.
Human cerebrum	3.50
Beef "	4.50
Sheep "	4.31
Dog "	3.60

While these values do not give an accurate idea of the lipase-content of the different brains, they nevertheless show that all have the ability to split triacetin to a marked extent.

b. DISCUSSION OF RESULTS:

Bacterial action has been avoided in all cases by the use of strong antiseptics. The natural oil of mustard has been most commonly used and has proven very satisfactory. Toluol gave good results also, but because of its weaker power of penetration, and the fact that it is less miscible with water, it was deemed less desirable than oil of mustard. But with the assurance that there was no bacterial action, the results in the case of lipase did not seem to be accurate beyond one-tenth of a cubic centimeter of tenth normal sodium hydroxide, and tests for other enzymes apparently had about the same degree of accuracy. This amount of variation would be found in solutions prepared in identically the same way, so that the methods were apparently unreliable beyond that margin.

With regard to the lipase, it is soluable in all the extractives used, but is seemingly much more soluable in some than in others. However, it may be that the different amounts of activity obtained from different extractives is due entirely to the surrounding media; that is, that the extractive itself, or some compound extracted from the brain by it, inhibits or accelerates the action of the lipase, and this possibility receives supporting evidence from the fact that the carbonate extract shows greater activity when rendered slightly acid.

One would expect the freezing method to give the best results, since by it the tissue is kept at a low temperature, and the chances of the enzyme deteriorating are much reduced. In fact, greater activity was obtained from tissue so prepared than from any other, with the exception of the air-dried tissue from which the lipoids had been extracted with benzene. If we may argue from

analogy, it follows that the frozen tissue would have given still higher activity, if its lipoids had first been extracted.

The experiments on the different divisions of the brain show in general that larger amounts of lipase are contained in the grey matter than in the white. The cerebrum shows high activity with the mid-brain about equal to it in fat-cleaving power. It is doubtless true that the mid-brain contains more white matter than the cerebrum, and for this reason it seems probable that the large nuclei of the mid-brain are especially rich in enzymes. The high activity of the cerebellum can easily be explained on the ground of it's large grey matter content. Besides having a cortex of nerve cells, each lateral lobe contains a large nucleus, the dentate nucleus, and other smaller nuclei are found throughout the interior of the cerebellum. It is not to be expected that the corpus callosum should surpass the medulla in enzyme activity, since the corpus callosum is composed entirely of white matter. However, in removing the corpus callosum, some grey matter was obtained and this may have increased its lipolytic power. A part of it's activity may also be explained by it's proximity to the cerebrum, since the enzymes contained in the cerebral cortex may, to some small extent at least, diffuse to the surrounding tissue after death.

A great amount of variation was shown in the behavior of the different substrates. The brain lipase acted at a different rate on each fat or ester and those tested behaved differently under the influence of an accelerator. These facts would indicate a specificity of the lipase toward a certain group of fats, but it's high activity on triacetin and monoacetin is doubtless a mat-

ter of chance and does not follow because these compounds are present in the nervous system.

It is rather remarkable that the proteases should show such a wide range of activity. That they sould attack nuclein is to be expected, since nucleoprotein, of which nuclein is a decomposition product, is present in large quantities in the nervous system. The action of the proteolytic enzymes on Wittes' peptone and possibly on egg albumen is also to be expected. But that they should attack such compounds as gelatin and casein is rather remarkable.

One would naturally think, as Wroblewski[10] expressed it, that the amylase was present for the purpose of splitting glycogen, yet no activity on glycogen could be detected. But the amylase was effective in splitting the complex carbohydrate, dextrin, so that it appears probable that under proper conditions glycogen would also be hydrolyzed. The activity of the amylase on arbutin and solol is doubtless a matter of coincidence, since neither of these compounds is known to exist in nerve tissue.

Vernon,[8] in a series of careful experiments, showed that the brain and many other tissues contain an oxidase which possesses the power of producing indophenol from alpha naphthol, paraphenylene-diamine and sodium carbonate. Other investigators have agreed upon the presence of a peroxidase in nerve tissue. Hence it seems certain that the proper conditions for the detection of both of these enzymes were not obtained. This possibility is always open in searching for any enzyme where negative tests have been obtained, since each enzyme requires a particular set of conditions and is easily affected by other compounds, the nature of the

substrate, and physical conditions. Hence, many enzymes, which at present are undetected in the nervous system, will doubtless be discovered in the future under more careful methods. The conclusion which can be drawn from these results, however, is that with the tests used, and with the conditions under which they were used, no enzymes are detectable.

V SUMMARY.

1. The brain contains a lipase, erepsin, a pepsin or trypsin like enzyme, or both, an amylase, a catalase, and enzymes which decompose arbutin and salol.

2. The lipase and proteases are best extracted by a mixture of glycerin and water, or by water.

3. Extracts of tissue, dried either by the freezing or air current method, showed greater activity than extracts of fresh tissue.

4. The highest activity was obtained from tissue extracted after drying and treating with benzene to remove the lipoids.

5. In general, the grey matter is more active than the white. Higher activity was obtained with extracts of the cerebrum, cerebellum, or mid-brain than with extracts of the medulla, or corpus callosum.

6. Triacetin and monoacetin form the best substrates for the action of the lipase. Lecithin and kephalin are also acted upon by it.

7. The amount of activity of the lipase as has been shown for other enzymes, is increased directly with the amount of enzyme, the amount of substrate, and the time during which the enzyme is allowed to act.

8. The amount of activity of the lipase is increased by the presence of sodium glycocholate, saponin, or a mixture of the mono- and dibasic phosphates of sodium.

9. The lipase acts best in a slightly acid medium, the erepsin in an alkaline medium.

10. Negative results were obtained in tests for peroxidase,

oxidase, reductase, guanase, urease and rennin.

11. Human, beef, sheep and dog brains all contain lipase.

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